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Changes in the Enzymes for Fatty Acid Synthesis and Desaturation during Acclimation of Developing Soybean Seeds to Altered Growth Temperature

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ABSTRACT

Temperature-induced changes in the enzymes for fatty acid synthesis and desaturation were studied in developing soybean seeds (*Glycine max* L. var Williams 82). Changes were induced by culture of the seed pods for 20 hours in liquid media at 20, 25, or 35°C. Linoleoyl and oleoyl desaturases were 94 and 10 times as active, respectively, in seeds cultured at 20°C as those cultured at 25°C. Both desaturases had negligible activity in seeds cultured at 35°C compared to seeds cultured at 20°C. Though less dramatic, other enzymes also showed differences in activity after 20 hours in culture at 20, 25, or 35°C. Stearoyl-acyl carrier protein (ACP) desaturase and CDP-choline:diacylglycerol phosphorylcholine transferase were most active in preparations from 20°C cultures. Activities were twofold lower at 25°C and a further threefold lower in 35°C cultures. Cultures from 25 and 35°C had 60 and 40%, respectively, of the phosphorylcholine:CTP cytidylyl transferase activity present in cultures grown at 20°C. Fatty acid synthetase, malonyl-coenzyme A:ACP transacylase, palmitoyl-ACP elongation, and choline kinase were not significantly altered by culture temperature. These data suggest that the enzymes for fatty acid desaturation and phosphatidylcholine synthesis can be rapidly modulated in response to altered growth temperatures, while the enzymes for fatty acid synthesis and elongation are not.

Alterations in fatty acid composition and content are a well documented phenotypic response of plants to alterations in environmental temperatures (24). Growth of plants at suboptimal temperatures results in increased lipid/protein ratios and an increase in the mole percent polyunsaturated fatty acid (24, 30), whereas growth at elevated temperatures induces the opposite effects. Though this response has been found in all classes of plants, little is known about the biochemical mechanisms responsible for these changes. Studies on the algae *Dunaliella salina* have shown that rapid changes occur in the activity of acyl-lipases followed by longer term changes in fatty acid desaturase activities (20, 21). Fatty acid desaturases were also implicated in the response of soybean tissue cultures to high and low growth temperatures (14, 15). Though desaturase activities were not measured in these studies, analyses of *in vivo* labeling patterns from cultures grown at 15 or 35°C clearly indicated the rate of fatty acid desaturation changed with culture temperature. However, only one systematic study of the changes in a synthetic pathway has been reported (11, 12). That study on lipid metabolism in cold adapted rye roots showed that synthesis of phosphatidylcholine increases during cold acclimation, with a net increase in the total content of phosphatidylcholine. Further experiments indicated that phosphorylcholine:CTP cytidylyl transferase was the rate limiting step and may control the changes found in phosphatidylcholine levels during cold acclimation of rye roots (11).

In the study presented here, the enzymes for synthesis and desaturation of fatty acids in soybean seeds were examined to identify which enzymes were changed during acclimation to altered growth temperatures.

MATERIALS AND METHODS

Materials

Cytidine 5′-diphospho-[methyl-^{14}C]choline, [1-^{14}C]stearic and palmitic acids, and [2-^{14}C]malonyl-CoA were obtained from Amersham Corp. All other ^{14}C and ^{3}H labeled substrates were purchased from NEN Research Products. HPLC solvents were purchased from American Burdick and Jackson. All other reagents were purchased from Sigma Chemical Co. or Research Organics Inc. Late log *Escherichia coli* B cells, from Grain Processing Corp., were used for the preparation of ACP[1] and acyl-ACP synthetase. The method of Rock and Cronan (25) was used for the preparation of ACP. Acyl-ACP synthetase was purified by a modification of the procedure of Rock and Cronan (26). Stearoyl- and palmitoyl-ACP were synthesized from the respective ^{14}C-fatty acids using the purified ACP and acyl-ACP synthetase described above (27).

Plant Materials

Soybeans (*Glycine max* [L.] Merr. var Williams 82) were grown in a greenhouse with a 28°C day/25°C night thermoperiod. Developing seed pods (stage R5 [4], 5–6 mm seeds) were collected from the plants, and cultured *in vitro* for 20 h at 20, 25, or 35°C (22). The seeds were harvested, weighed,

[1] Abbreviation: ACP, acyl carrier protein.

homogenized, and fractionated using a modification of the procedure of Lord *et al.* (13). Subcellular fractions were obtained by differential centrifugation. The total homogenate was centrifuged for 10 min at 420*g* to remove cellular debris, and the supernatant was centrifuged at 8,000*g* for 3 min in a Beckman TL ultracentrifuge to pellet the chloroplasts. The supernatant was further centrifuged for 45 min at 104,000*g* to pellet the remaining membranous material (post-chloroplast membranes). The pellets were resuspended in homogenization medium (13) at 1 mL/g fresh weight. These fractions were then used for all enzyme assays. Fatty acid synthetase, malonyl-CoA:ACP transacylase, stearoyl-ACP desaturase, and palmitoyl-ACP elongation were assayed in the chloroplast fraction. Phosphorylcholine:CTP cytidylyl transferase was assayed in the 8,000*g* supernatant. CDP-choline:diacylglycerol phosphorylcholine transferase, oleoyl, and linoleoyl desaturases were assayed using the post-chloroplast membrane fraction. Choline kinase was assayed in the 104,000*g* supernatant.

Enzyme Assays

Enzyme preparations from seeds grown at the three culture temperatures were assayed under identical conditions in all cases. All results were the average of duplicate assays on 3 to 7 different preparations. The chloroform soluble substrates and products from assays for palmitoyl-ACP elongation and for stearoyl, oleoyl, and linoleoyl desaturation were derivatized with nitrophenylhydrazine and separated by HPLC (18). Radioactivity and mass were quantitated by simultaneous monitoring of the HPLC effluent for absorbance (400 nm for nitrophenyl esters) and radioactivity (by flow through scintillation counting). Malonyl-CoA:ACP transacylase was measured at 20°C using the method of Guerra and Ohlrogge (5). Fatty acid synthetase was assayed at 30°C by the method of Shimakata and Stumpf (28). Fatty acids from these assays were isolated by TLC and quantitated by liquid scintillation counting of the resulting fractions. Palmitoyl-ACP elongase was assayed at 25°C using the method of Jaworski *et al.* (7) and quantitated as described above. The method of McKeon and Stumpf (17) was used, at 25°C, to assay the desaturation of stearoyl-ACP to oleoyl-ACP. Assays for oleoyl- and linoleoyl-CoA desaturases were conducted at 25°C using the procedure of Martin and Rinne (16). Choline kinase was assayed at 30°C using the method of Kinney and Moore (9). Assays were also performed at 30°C for phosphorylcholine:CTP cytidylyltransferase using a modification of the method of Choy *et al.* (2). Finally, CDP-choline:diacylglycerol phosphorylcholine transferase was measured at 30°C using the procedure of Moore (19).

RESULTS AND DISCUSSION

The enzymes of fatty acid synthesis and desaturation and of phosphatidylcholine synthesis (the substrate for fatty acid desaturases in sobyeans [6, 16]) were surveyed as a first step in determining the mechanism for environmental regulation of fatty acid metabolism (Fig. 1). Experiments using soybean cell suspension cultures indicated cultured cells respond to changes in growth temperature more rapidly than whole plants (14, 15). However, the rate of lipid synthesis in these cultures is much lower than that in developing seeds. Systems for culturing intact pods and seeds have been described for the study of soybean seed development (22). The data presented in this paper show that cultured seeds respond rapidly to changes in growth temperatures while retaining the high rates of lipid synthesis found on the plant. Analyses of developing seeds indicated that desaturase activities and lipid synthesis were maximal in the early developmental stages of R5 to R6 (1, 3). Therefore, developing (R5) soybean seeds were chosen as a model system for these studies because of the combination of a high rate of lipid synthesis in this tissue and the availability of an *in vitro* culture system.

Fatty Acid Synthesis and Elongation

The first step in the synthesis of fatty acids is catalyzed by acetyl-CoA carboxylase. This enzyme is known to be under complex regulatory control (29). Little is known, however, about the regulation of other enzymes involved in the synthesis and desaturation of fatty acids. When the enzymes for fatty acid synthesis and elongation were assayed, only minor changes in activity were detected after 20 h (Fig. 2). Malonyl-CoA:ACP transacylase showed only a 20% change in activity between 20 and 35°C cultures. Fatty acid synthetase activities (Fig. 2B) were greatest in cultures from 25°C, while cultures from 20 and 35°C were approximately 60 and 50% as active, respectively. However, such changes are relatively minor compared to other enzymes in the pathway. This observation, coupled with the relatively high activities of the two enzymes (Table I), suggests they are probably not responsible for the change in lipid composition. Though palmitoyl-ACP elongation also showed little change after 20 h of acclimation (Fig. 2C), this enzyme complex has one of the lowest total activities and therefore has the potential to control flux through the pathway under all conditions.

Fatty Acid Desaturation

The activities of stearoyl, oleoyl, and linoleoyl desaturases were all dramatically altered by changes in growth temperature (Fig. 3). These modulations were not caused by alterations in the recovery of the subcellular fractions after culture at 20, 25, and 35°C, as shown by the fact that of the four chloroplastic enzymes surveyed only stearoyl-ACP desaturase activity changed with changes in growth temperature. This conclusion was supported by a test of protein recoveries in the various subcellular fractions. At all three growth temperatures, the chloroplast fraction contained 2.1 to 2.3 mg of protein per g fresh weight. The same results were found with the total homogenate, postchloroplast membrane, and 104,000*g* supernatant fractions (35–36, 3.9–4.0, and 26–28 mg/g fresh weight, respectively, at all culture temperatures). The total activity of stearoyl-ACP desaturase decreased twofold between 20 and 25°C and sixfold between 20 and 35°C cultures (Fig. 3A). The largest changes occurred in oleoyl and linoleoyl desaturases. Oleoyl desaturase activity decreased from 4.7, in 20°C cultures, to 0.46 nmol/min/mL in cultures grown at 25°C, and further decreased to about 0.002 in 35°C

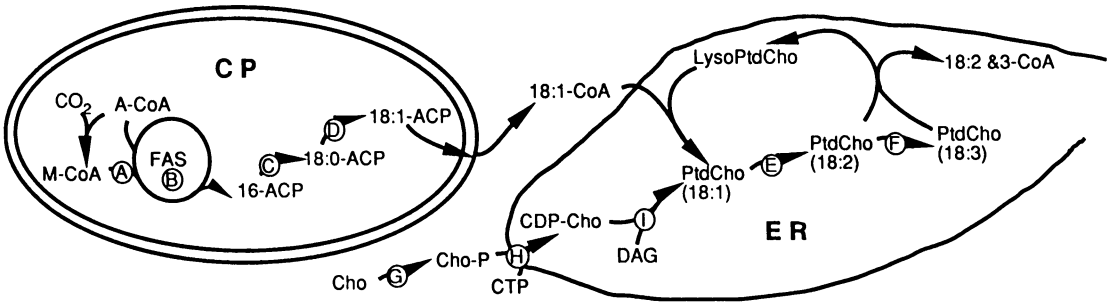

Figure 1. Simplified diagram of the reactions surveyed. This diagram illustrates soybean metabolism in terms of the enzymatic steps analyzed and the subcellular fractions used. Abbreviations are: M-CoA, malonyl-CoA; A-CoA, acetyl-CoA; FAS, fatty acid synthetase; C P, chloroplast; ACP, acyl carrier protein; 16, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; Cho, choline; Cho-P, phosphorylcholine; CDP-Cho, cytidine diphosphocholine; DAG, diacylglycerol; PtdCho, phosphatidylcholine; and LysoPtdCho, lysophosphatidylcholine. The enzymes surveyed are: A, malonyl-CoA:ACP transacylase; B, fatty acid synthetase; C, palmitoyl-ACP elongation; D, stearoyl-ACP desaturase; E, oleoyl desaturase; F, linoleoyl desaturase; G, choline kinase; H, phosphorylcholine:CTP cytidylyl transferase; and I, CDP-choline:diacylglycerol phosphorylcholine transferase.

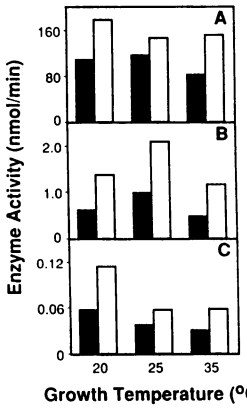

Figure 2. Effect of growth temperature on the activities of enzymes for fatty acid synthesis and elongation. Developing soybean pods (stage R5) were harvested from greenhouse grown plants and cultured for 20 h, in liquid media, at the temperatures indicated. The enzymes were assayed in isolated chloroplasts prepared by differential centrifugation of whole seed homogenates. Chloroplast preparation from all three conditions were assayed under identical conditions. Enzymes were assayed as described in "Materials and Methods." Closed bars are total activity in nmol/min/mL, open bars show specific activity in nmol/min/mg protein. A, Malonyl-CoA: ACP transacylase; B, fatty acid synthetase; C, palmitoyl-ACP elongation.

Table I. *Relative Activities for the Enzymes of Fatty Acid Synthesis and Desaturation as a Function of Growth Temperature*

The activities are based on the total activities of each enzyme when assayed as described in "Materials and Methods." For each growth temperature the activities are expressed as a percent of the fatty acid synthetase activity at 20°C. Abbreviations are: M-CoA trans., malonyl-CoA:ACP transacylase; FAS, fatty acid synthetase; 16-ACP elong., palmitoyl-ACP elongation; 18:0 desat., stearoyl-ACP desaturase; 18:1 desat., oleoyl desaturase; 18:2 desat., linoleoyl desaturase; Cho kinase, choline kinase; CDP trans., phosphorylcholine:CTP cytidylyl transferase; Cho-P trans., CDP-choline: diacylglycerol phosphorylcholine transferase.

Enzyme	Growth temperature (°C)		
	20	25	35
	Relative activity %		
M-CoA trans.	17,900	19,100	13,700
FAS	100	160	78
16-ACP elong.	9	6	5
18:0 desat.	7	4	1
18:1 desat.	773	75	<1
18:2 desat.	1,410	23	2
Cho kinase	283	331	171
CDP trans.	929	535	359
Cho-P trans.	36	18	7

cultures. The same trend was seen with linoleoyl desaturase, which showed activities of 8.6, 0.14, and <0.02 nmol/min/mL in postchloroplast membranes derived from 20, 25, and 35°C cultures, respectively. Though detectable (*i.e.* 2–3 times background), the oleoyl and linoleoyl desaturase activities in preparations from 35°C cultures were so low that the absolute activities could not be determined accurately. These data are consistent with either a dramatic reduction in the levels of desaturase or an inactivation of the enzyme during culture at 35°C. These changes in desaturase activities were in agreement with earlier observations by MacCarthy and Stumpf (15) using *in vivo* labeling of soybean cell suspension cultures. Oleoyl and linoleoyl desaturases have relatively high activities in preparations from 20°C cultures (Table I) and become rate limiting in 35°C cultures, while stearoyl-ACP desaturase activity is relatively low in preparations from all three growth regimes. From these data, it is apparent that any or all of the desaturases have the potential to be regulatory sites in the pathway.

Phosphatidylcholine Synthesis

Only minor changes in activity were found in the enzymes catalyzing the first two steps in the conversion of choline to phosphatidylcholine (Fig. 4). Choline kinase showed maximum activity in 105,000*g* supernatants from 25°C cultures. Kinase was 85 and 50% as active in preparations from 20 and 35°C, respectively (Fig. 4A). Phosphorylcholine:CTP cytidylyl transferase was most active in cultures grown at 20°C and

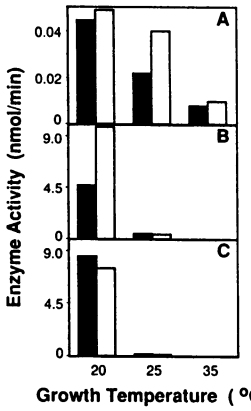

Figure 3. Fatty acid desaturase activities after growth at high normal and low temperature. Subcellular fractions were prepared from stage R5 seeds after 20 h in culture at 20, 25, or 35°C. Assays were performed as described in the text. A, Stearoyl-ACP desaturase; this enzyme was assayed using isolated chloroplasts; B, oleoyl-CoA desaturase; C, linoleoyl-CoA desaturase. Oleoyl and linoleoyl desaturases were measured in the postchloroplast membrane fraction. Closed bars show total activity in nmol/min/mL, open bars indicate specific activity in nmol/min/mg protein.

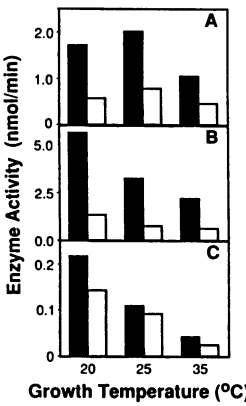

Figure 4. Effect of growth temperature on the enzymes for the synthesis of phosphatidylcholine from choline. Postchloroplast membranes and the 104,000*g* supernatants were prepared as described in the text. A, Choline kinase. Kinase activities were measured in 104,000*g* supernatants. B, phosphorylcholine:CTP cytidylyl transferase. C, CDP-choline:diacylglycerol phosphorylcholine transferase. Enzyme B was assayed in the 8,000*g* supernatant and enzyme C in the postchloroplast membrane fraction. Closed bars show total activity in nmol/min/mL, open bars indicate specific activity in nmol/min/mg protein.

decreased in activity with increasing culture temperatures (Fig. 4B). Compared to other enzymes in this study, the change in total activity was not large (*i.e.* 5.7 *versus* 2.2 nmol/min/mL at 20 *versus* 35°C). Phosphorylcholine:CTP cytidylyl transferase is reported to be either soluble or membrane bound depending on the plant examined (10, 23). The soybean enzyme was found in both the postchloroplast membrane and 105,000*g* supernatant fractions. The ratio of activities in the two fractions changed with changes in culture temperature (data not shown). CDP-choline:diacylglycerol phosphorylcholine transferase showed significant changes in activity in preparations from the three growth regimes (Fig. 4C). The enzyme activity decreased twofold in 25 *versus* 20°C cultures and a further threefold in postchloroplast membranes from 35°C cultures. CDP-choline:diacylglycerol phosphorylcholine transferase had relatively low activities at all temperatures, while choline kinase and phosphorylcholine:CTP cytidylyl transferase had high activities in all cases. Thus, only the former appears to be a possible site for control of phosphatidylcholine synthesis in this system. These findings differ from those for rye roots where cytidylyl transferase is the rate limiting step (11).

SUMMARY

From these studies, it is apparent that the activities of several enzymes involved in the synthesis and desaturation of fatty acids are modulated in response to changes in environmental temperature. These results are consistent with previous observations showing temperature induced changes in the content of unsaturated fatty acids in plants (15, 24, 30). Four of the nine enzymes surveyed showed significant changes in activity induced by altered growth temperature.

Two enzymes, stearoyl-ACP desaturase and CDP-choline:diacylglycerol phosphorylcholine transferase, as well as palmitoyl-ACP elongation, showed limiting activities *in vitro* when compared to the other enzymes surveyed (Table I). The combination of modulation by growth temperature (Figs. 3A and 4C) and low total activity *in vitro* suggest that stearoyl-ACP desaturase and CDP-choline:diacylglycerol phosphorylcholine transferase have the potential to be control sites *in vivo*. However, without additional data on substrate pool sizes and metabolic flux through the pathway, their physiological role in regulation can not be assessed (8). Four of the five of the enzymes found to be modulated are membrane bound, thus a number of mechanisms exist which could give rise to these data. Because all assays were run under identical conditions, these data cannot distinguish between most of the different mechanisms which may give rise to the changes seen. We can conclude that changes in O_2 concentration are not responsible for changes in desaturase activity, since all assays were run at the same temperature. However, these studies cannot distinguish between a change caused by alteration of the enzymes and a change caused by alteration of the membrane environment of these enzymes. More detailed experiments on individual enzymes are currently in progress to differentiate these possibilities.

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